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EARLY DETECTION OF DENGUE INFECTIONS USING CLUSTER SAMPLING AROUND INDEX CASES

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Abstract. A two-year study using a cluster investigation method was conducted in West Jakarta, Indonesia to demonstrate the detection of dengue cases prior to onset of clinical illness. The clusters consisted of family members and neighbors of 53 hospitalized dengue index cases. Among 785 adult and child volunteers enrolled, 17 (2.2%) post-enrollment dengue (PED) infections were identified. Eight PED cases were asymptomatic and nine were symptomatic. Symptomatic cases included eight with dengue fever and one with dengue hemorrhagic fever (DHF) (grade II). Among the eight asymptomatic PED cases, viremia was detected in two. Eleven volunteers had acute dengue infections at the time of enrollment. Four of the 11 developed DHF, resulting in a total of five DHF cases detected during the investigation. This study design can serve as a benchmark for future investigations that seek to define early immunologic events following dengue infections that contribute to the development of DHF.

INTRODUCTION

Infection with any of the four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) can be asymptomatic, result in a mild-to-moderate febrile illness termed dengue fever (DF), or a more severe illness characterized by bleeding and shock called dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).[1,2] After an initial infection with dengue virus, homologous serotype immunity persists for many years; however, individuals remain susceptible to infection with the remaining dengue virus serotypes. In several studies, DHF occurs 15–80 times more frequently in individuals experiencing a second dengue virus infection compared with individuals experiencing their first infection.[3] The biologic events that lead to a higher frequency of DHF in secondary dengue virus infections compared with primary infections are not completely known.

Antibody-dependent enhancement of dengue virus infection leading to increased viral load is a leading theory for severe diseases caused by dengue virus. Increased levels of circulating dengue virus have been documented in DHF cases compared with DF cases.[4–6] However, the immunologic events that occur in the early stages of infection that lead to severe disease have not been characterized in patients. In the absence of an animal model for DHF, researchers are forced to try to characterize early immunologic events in patients with recently acquired dengue virus infections. A major limiting factor in such an investigation is identifying individuals prior to the onset of their clinical illness so that early events can be characterized. The current study was designed to overcome that problem by identifying individuals very early in the infection. Family members and nearest neighbors of index cases hospitalized with acute dengue virus infections were enrolled and followed longitudinally to determine the incidence rate of dengue virus infection. This proof-of-concept study was conducted to evaluate the hypothesis that monitoring persons clustered around an index case would be an efficient and practical way to obtain blood samples before, during, and after dengue infection.

MATERIALS AND METHODS

Study location and population. West Jakarta (West Java), Indonesia, was selected as the location to pilot the concept of a dengue cluster study for several reasons. The catchment area for cases is in a tropical, urban setting with approximately 2.5 million inhabitants of various socioeconomic strata, mostly of low income. On average, 5–10 persons may live in a single housing unit measuring 10×15 meters^2. Most neighborhoods are densely populated, with dwellings in relatively close proximity to one another (approximately 10 meters apart) and constructed of cement and/or wood. The average house does not have piped water and some communities share toilet facilities. The government supplies potable water in large cans and water for bathing is often stored in a traditional uncovered, tub-like area (bak mandi) located in bathrooms. The bak mandi has long been a potential source of *Aedes* mosquito larvae,[7] in addition to other open containers used for water storage and various other miscellaneous items (i.e., flower pots, tires) containing water found in close proximity to human dwellings.[8] *Aedes aegypti* mosquitoes are ubiquitous throughout Indonesia and transmission of all four dengue virus serotypes is known to occur. According to the Indonesian Communicable Disease Control, the three-year incidence of dengue disease in Jakarta was 75.4, 52.4, and 108.5 cases per 100,000 population from 2001 to August 2003, respectively (unpublished data).

Dengue virus laboratory tests. Serum samples were tested for the presence of IgM antibodies to dengue virus using commercial enzyme-linked immunosorbent assay (ELISA) kits (Focus Technologies, Cypress, CA). Hemagglutination inhibition (HI) assays and plaque reduction neutralization tests (PRNTs) were also performed to confirm dengue virus infection and to classify the infection as primary or secondary based on the antibody response.[9] Dengue virus RNA in blood was detected using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay.[10] Blood samples collected from symptomatic volunteers during the acute stage of febrile illness and from suspected asymptomatically infected volun-

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teers were tested for virus by culture in C6/36 cells, and virus was identified using serotype-specific monoclonal antibodies for dengue virus.[11]

Study design. Each cluster of volunteers was identified by an index case from the pediatric ward at Sumber Waras Hospital in West Jakarta. Physicians at this local community hospital have a well-established relationship with the surrounding community and are experienced in clinically diagnosing dengue virus infections. For the index cases, children 4–14 years of age were recruited. Ward clinicians were asked to select 1–2 cases per week based on clinical manifestations, together with the detection of IgM antibody to dengue virus by ELISA and/or dengue viral RNA by RT-PCR. In most cases, these diagnostic assays were performed within 48 hours of index case identification. Other considerations in selecting index cases included automobile accessibility to their community and the willingness of the family to participate.

Usually within 48 hours of index case identification, family members and nearest neighbors greater than four years old who lived within a 10-meter radius of the index case's home were invited to participate. Volunteers who were afflicted with or who had a history of severe anemia, bleeding disorder, or any known immunologic disorder were excluded from the study. On enrollment, we measured the otic temperature, and collected blood samples and demographic data from each study volunteer. Volunteers were followed for 14 consecutive days for fever and other clinical manifestations suggestive of acute dengue virus infection. During the monitoring period, we collected additional blood samples from each volunteer every 2–3 days. We also collected blood specimens from each volunteer who developed a fever or dengue-like signs or symptoms and processed them for a diagnosis of dengue. To confirm that dengue virus was the cause of fever, we tested samples within 24–48 hours of collection for dengue viral RNA by RT-PCR and for IgM antibody to dengue virus by ELISA. If a sample collected from a febrile volunteer was positive for IgM antibodies to dengue virus, the enrollment blood sample for that volunteer was tested for IgM antibodies to dengue virus to determine if seroconversion had occurred. These laboratory tests were performed at the Virology Laboratory of the U.S. Naval Medical Research Unit No. 2. Blood samples treated with EDTA were also obtained at the onset of fever and sent to Sumber Waras Hospital for hematocrit and platelet count determinations.

Febrile volunteers with a positive IgM ELISA or RT-PCR result were encouraged to be hospitalized for close monitoring and serial laboratory tests. Volunteers refusing hospitalization were monitored closely as outpatients until they were afebrile for two consecutive days. Convalescent blood samples were obtained two weeks later and again at six months.

Hospitalized volunteers were bled daily until two days after defervescence, two weeks later, and approximately six months after discharge from the hospital. Blood samples were routinely tested for hemoglobin, hematocrit, platelets, white blood cells, protein, and albumin. A tourniquet test was done on admission and daily ultrasound examinations for ascites and pleural effusions were performed until two days after defervescence to detect evidence of plasma leakage. We classified hospitalized volunteers as DF or DHF (World Health Organization, 1997)[12] following evaluation of the clinical data.

We categorized all volunteers clustered around the index cases into one of three groups: non-dengue (ND) infection, dengue infection at enrollment (ED), or post-enrollment dengue (PED) infection. A PED case was defined as a volunteer who developed fever after enrollment and demonstrated seroconversion for IgM antibodies to dengue virus and/or dengue viremia by RT-PCR or virus isolation. Volunteers who developed fever but were negative for IgM antibodies to dengue virus or viremia were later classified as PED infections if there was a four-fold increase in the HI antibody titer to dengue virus between enrollment and the two-week post-enrollment blood samples. For equivocal HI results (less than a four-fold increase in titer), pre-enrollment and two-week post-enrollment $PRNT_{50}$ titers were compared for a $\geq$ four-fold increase against one or more dengue virus serotypes. For volunteers who never developed fever, laboratory evaluation of serum samples (dengue serology and virus detection) was still conducted throughout the 14-day monitoring period. Asymptomatic volunteers who were positive for viremia and/or showed a four-fold increase in HI titer between the enrollment and two-week post-enrollment blood samples were also classified as PED cases.

Volunteers that demonstrated evidence of infection (IgM, RT-PCR, or virus isolation), with or without symptoms at the time of enrollment were classified as ED cases. All other volunteers were classified as ND cases.

The U.S. Naval Medical Research Unit No. 2 Institutional Review Board and the Indonesian Ministry of Health Ethical Review Committee reviewed and approved the human use protocol (DoD# 30861) used in the study, in compliance with all U.S. federal regulations governing the protection of human subjects. Informed written consent was obtained from all participants, and if < 15 years of age, from a parent or legal guardian.

RESULTS

Over a two-year period from October 2001 to October 2003, 53 confirmed dengue index cases were identified in the pediatrics ward at Sumber Waras Hospital (Table 1 and Figure 1). Infections by all dengue virus serotypes were identified among the index cases. The predominant serotype identified was DEN-1 (10), followed by DEN-2 (8), DEN-3 (5), and DEN-4 (2). The serotype was not determined in 28 index cases. None of the index dengue infections was fatal.

From these 53 dengue index cases, 53 cluster investigations were performed where family members and nearest neigh-

TABLE 1

Demographics of volunteers and corresponding dengue infections identified in West Jakarta, Indonesia*

	Number of index cases	Community volunteers	ED cases	PED cases
Total	53	785	175	17
Males:females	23:30	317:468	84:91	8:9
Adults	NA	453	87	8
Children (4–14 years old)	53	332	88	9
Mean age (years)	6.6	22.57	19.2	20.2
Family members	NA	164	32	2
Nearest neighbors	NA	621	143	15

* ED = enrollment dengue cases; PED = post-enrollment dengue cases; NA = not applicable.

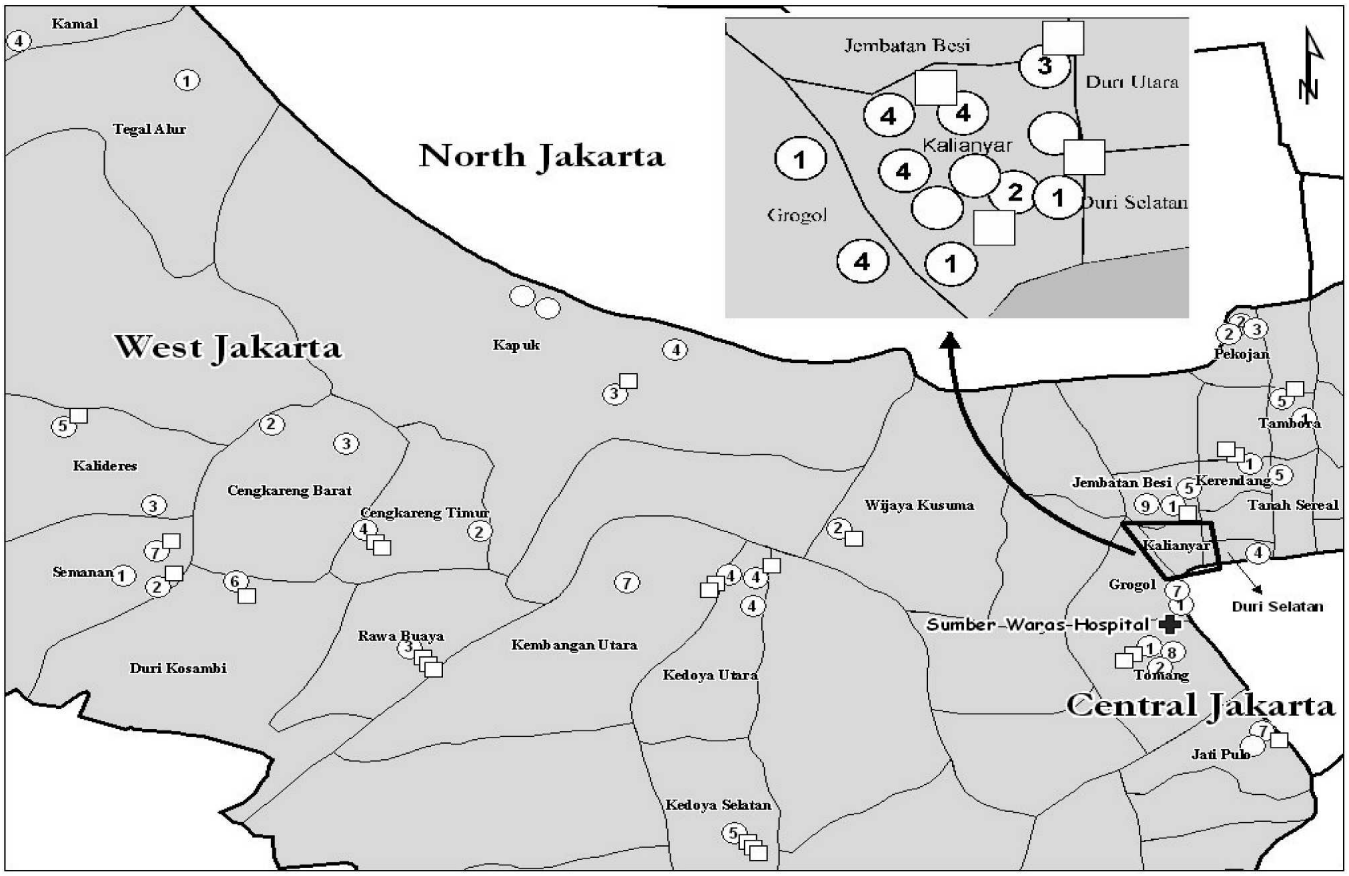

FIGURE 1. Map of index cases and corresponding dengue infections identified in respective clusters in West Jakarta, Indonesia. Each circle represents an index case. Each square represents an acute dengue case identified at enrollment by detection of virus (ED acute infection) or during a two-week period of monitoring by detection of virus or by seroconversion (post-enrollment dengue infection). The numbers inside the circles represent convalescent dengue infections detected by the presence of IgM antibody only at enrollment (ED convalescent infection). The study hospital (Sumber Waras Hospital) is indicated.

bors surrounding the residence of each index case were monitored for dengue virus infection. A total of 785 volunteers were recruited from the 53 community clusters, of which 541 completed the entire 14-day monitoring period. The 244 volunteers who did not complete the entire monitoring period were included in the analysis since at least one blood specimen was available for evaluation from each volunteer. Table 1 shows the demographics of the volunteers in the study.

Among the 785 cluster volunteers, 175 (22.3%) were classified as ED cases. Table 1 also shows the demographics of ED cases. Eleven ED cases were found to be viremic for dengue at enrollment by the RT-PCR. The DEN-1 serotype was identified in three cases and DEN-2 in eight cases. Confirmatory virus isolation was accomplished in 5 of the 11 cases. The remaining 164 ED cases had evidence of recent dengue virus infection, as indicated by IgM antibodies to dengue virus at enrollment and were classified as convalescent infections (Figure 1).

Nine of the 11 viremic ED cases had fever at enrollment. The nine febrile volunteers were hospitalized for close monitoring within 24–48 hours of enrollment after dengue serologic and/or RT-PCR results were known. Four of the febrile hospitalized ED cases were subsequently diagnosed with DHF (three grade I and one grade II). All four DHF cases were caused by a DEN-2 virus. Analysis of HI and PRNT titers suggested that two of the DHF cases were secondary infections and two were primary infections. The volunteers with primary infections were 10 and 8 years old, whereas those with the secondary infections were 20 and 21 years old.

Seventeen (2.2%) volunteers were diagnosed as PED cases during the 14-day monitoring period (Table 2), and most (15 of 17, 88%) were among nearest neighbors (Table 1). Of the 17 PED cases, 10 were positive by the RT-PCR (1 DEN-1, 7 DEN-2, 1 DEN-3, and 1 DEN-4), and the serotype was confirmed by virus isolation in 5 cases. The other seven PED cases became positive for IgM antibodies to dengue virus during monitoring, and dengue infections were also evident by serocoversion ($\geq$ 4-fold increase in antibody titer) by HI or PRNT. Four of the 17 PED cases were primary infections based on HI and $PRNT_{50}$ results, and all others were secondary infections.

Nine of the 17 PED cases experienced symptomatic illnesses. Eight of the nine were DF and one was diagnosed as DHF grade II. The HI and PRNT patterns comparing preillness and convalescent blood samples suggested a secondary infection in all but three cases (Table 2).

The one PED DHF case was hospitalized five days after enrollment with a fever of 38.8°C and a positive tourniquet test result, along with a history of epistaxis prior to admission that continued for two days while hospitalized. Small pleural

TABLE 2
Characteristics of 17 post-enrollment dengue (PED) infection cases identified

Study no.	Age (years)/sex	DEN serotype	Clinical classification*	Serologic response
CL 0102	9/F	4†	DF	Secondary
CL 0118	18/M		Asymptomatic	Secondary
CL 0417	37/F		Asymptomatic	Secondary
CL 0710	14/F		Asymptomatic	Secondary
CL 1002	7/M	2	DF	Primary
CL 1211	5/M		DF	Secondary
CL 1514	30/F	2	DF	Secondary
CL 1806	10/F	2†	DHF grade II	Secondary
CL 2202	8/M	2†	DF	Primary
CL 2204	40/F	2†	Asymptomatic	Secondary
CL 2408	47/F	1	Asymptomatic	Secondary
CL 3213	24/M	2†	DF	Primary
CL 3716	9/M		Asymptomatic	Secondary
CL 3911	32/F	2	DF	Secondary
CL 4202	11/M	3	DF	Secondary
CL 5115	36/M		Asymptomatic	Secondary
CL 5304	7/F		Asymptomatic	Primary

* DF = dengue fever; DHF = dengue hemorrhagic fever.
† Positive by both dengue virus reverse transcriptase–polymerase chain reaction and virus isolation in C3/36 cells.

effusions and ascites developed on day three, but resolved by day five. The platelet count was normal on admission but decreased to 40,000/mm^3 by hospital day six. From admission, total protein and albumin levels decreased from 8.1 g/dL to 5.2 g/dL and from 3.5 g/dL to 2.9 g/dL, respectively (normal levels: protein = 6.4–8.7 g/dL and albumin = 3.5–5.2 g/dL) on day six. The patient remained hemodynamically stable throughout hospitalization and was discharged on day 10.

Asymptomatic dengue virus infections occurred in eight of the PED cases (Table 2). Dengue viremia was detected in two of the eight cases, with DEN-1 identified in one case by RT-PCR and DEN-2 identified in the second case by both RT-PCR and virus isolation. In the case with DEN-1, virus was detected in the blood sample obtained on day 10. For the case with DEN-2, virus was detected in the blood sample obtained on day 4. To our knowledge, these cases, together with the two asymptomatic ED viremic cases mentioned earlier, represent the first documentation of asymptomatic dengue viremia in human volunteers from naturally acquired infections.

Table 3 shows the serotype-specific relationship of dengue viruses identified from index and cluster cases. There were seven instances in which the virus was identified from both the cluster case and the corresponding index case (four ED cases and three PED cases). The infecting serotype of the cluster case corresponded with the infecting serotype of the index case in four instances (all DEN-2) and in three instances the infecting serotypes were different. We did not detect a specific pattern of the infecting serotype between the ED and PED cases.

DISCUSSION

Our cluster investigation method was designed as an alternative approach to the commonly used prospective cohort study method for investigating the natural history of dengue virus infection. Unlike the cohort study, the cluster investigation method allows the collection of clinical information and biologic samples during the early pre-infection stage. This allows for more precise characterization of the early immunopathologic events that contribute to either mild or severe clinical manifestations of dengue virus infection.

TABLE 3
Summary of known dengue virus (DEN) serotypes for the index case and corresponding cluster dengue cases (11 viremic ED cases and 17 PED cases)*

Cluster case ID no.	Cluster case serotype†	Index case serotype
ED cases		
CL 0602	DEN-2	DEN-1
CL 0906	DEN-2	DEN-2
CL 1001	DEN-2	DEN-3
CL 1101	DEN-2	DEN-2
CL 1105	DEN-2	–
CL 1108	DEN-2	–
CL 1201	DEN-2	–
CL 3701	DEN-1	–
CL 3702	DEN-1	–
CL 3815	DEN-2	–
CL 4007	DEN-1	–
PED cases		
CL 0102	DEN-4	–
CL 0118	–	–
CL 0417	–	–
CL 0710	–	DEN-2
CL 1002	DEN-2	DEN-1
CL 1211	–	–
CL 1514	DEN-2	–
CL 1806	DEN-2	DEN-2
CL 2202	DEN-2	–
CL 2204	DEN-2	–
CL 2408	DEN-1	–
CL 3213	DEN-2	DEN-2
CL 3716	–	–
CL 3911	DEN-2	–
CL 4202	DEN-3	–
CL 5115	–	–
CL 5304	–	DEN-4

* ED = enrollment dengue; PED = post-enrollment dengue; ID no. = subject identification number; – = negative result.
† Serotype identified by dengue virus reverse transcirptase–polymerase chain reaction and/or virus isolation.

Among 785 volunteers in this investigation, we observed 17 PED (new) dengue infections that were both symptomatic and asymptomatic, including one case of DHF. The calculated incidence rate of dengue infection was 567 cases per 1,000 person-years of follow-up. Based on the one observed DHF case, the calculated DHF incidence rate was 33 DHF cases per 1,000 person-years of follow-up, resulting in a DHF to DF incidence rate ratio of 1:18. This ratio closely approximates other epidemiologic observations, showing that DHF occurs in roughly 5% of dengue virus infections in areas where all four serotypes of dengue virus are endemic.[13–15]

We detected 175 individuals who had either a recent dengue virus infection or who were acutely infected at the time of enrollment and diagnosed with DHF. Eleven of the 175 volunteers were found to be viremic. Combining these 11 cases with the PED cases, a total of 28 acutely infected dengue cases were observed among the volunteers (Figure 1), resulting in a calculated incidence of 933 acute dengue cases per 1,000 person-years of follow-up. Of the 28 acute dengue cases, five were classified as DHF resulting in an overall DHF incidence of 166 cases per 1,000 person-years.

Virologic analysis of dengue cases confirmed that all four serotypes circulated in West Jakarta. The DEN-1 serotype was the predominant virus identified among the index cases, but DEN-2 caused most of the cluster cases, including all five

DHF cases. We plan to genetically characterize these viruses and compare them to other circulating Asian strains. Using banked sera, we will also examine the kinetics of infection in both symptomatic and asymptomatic cases by a quantitative RT-PCR. Although an association between the level of dengue viremia and severity of symptomatic disease was previously established, these analyses may provide some insight into whether there is a correlation between the level of viremia and symptomatic or asymptomatic dengue virus infection.

An attractive feature of the cluster investigation method is that it does not rely on outbreak events to accumulate a sizeable number of cases. When longitudinally following a randomly selected cohort, the study population may or may not experience outbreak-related dengue virus transmission, so that to arrive at a sufficient number of DHF and DF cases for statistical analysis, one may have to follow a particular cohort for many months to years. With the cluster investigation method that selects index cases as a reference point, only days to weeks of follow-up are required to define past and present virus activity in an area. A shorter follow-up period would significantly reduce cost and personnel needs.

Cohort studies to date have been unsuccessful in documenting asymptomatic viremia in humans from naturally acquired dengue infection.[16] Given the natural history of dengue virus infection, such silent viremia would seem plausible, but with the design of typical longitudinal cohort studies, infected volunteers are only identified upon the development of symptoms. Asymptomatic infections are only detected by seroconversion of blood samples collected at pre-determined time points. Using a cluster design approach, we were able to document eight asymptomatic dengue infections, two of which demonstrated viremia. To our knowledge, this is the first documentation of asymptomatic viremia in naturally acquired human dengue infections.

The one obvious disadvantage of the cluster method is that volunteers are required to participate in an intensive period of surveillance where, despite being healthy at the time, they are asked to donate blood samples every 2–3 days for a period of two weeks. We had little difficulty recruiting the requisite 10–15 people per cluster and often had to refuse additional enrollment of willing volunteers. With augmented resources and experience, we plan to expand our study design to new areas (East Jakarta and another major city in West Java, Indonesia) and to increase the number of index cases (6–8 per week) and persons recruited per cluster (up to 20). The major challenge to our cluster study design is maintaining adequate support (finances and trained personnel) for the intensive community monitoring. We believe that the yield of the cluster investigation method is equal to or substantially greater than that of other prospective study methods used to examine the epidemiology of naturally acquired dengue virus infection. As this method is applied to other countries with varying cultural sensitivities and expectations, differing levels of success may be encountered.

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